Laboratory Analysis of Proteins

Lecture Goal(s): To consider the characteristics of proteins that can be utilized for laboratory analysis. Currently used methods for analysis of proteins will be addressed.

Lecture Objectives: Upon completion of this class material each student will be able to do the following:

1. Cog/II Describe tests for protein determination that depend on the physical properties of proteins.

   a. mass or weight
   b. water competition effects
   c. UV absorption
   d. density
   e. insoluble complex formation
   f. pH, size, and molecular weight

2. Cog/II Describe tests for protein determination that depend on the chemical properties of proteins.

   a. Kjeldahl method
   b. peptide bond methods
      Folin Ciocalteau
      Biuret
      Lowry-Copper Phenol
   c. dye binding applications

In this section we will consider general analytical processes that are used in the evaluation of proteins. Some methods described below measure all proteins, whereas others measure groups of proteins or specific individual proteins. Also mentioned are methods that have been used in the past and methods that are currently being used in research settings to provide fundamental information on proteins that will be useful for the development, evaluation, and understanding of routine methods.

Tests Dependent on the Physical Properties of Proteins

Introduction

The various types of proteins in the body have different solubility coefficients; thus, they may be isolated by varying the salt and/or solvent concentration of their environment. As discussed, proteins are made up of chains of amino acids; depending on the length and type of chain, they vary in charge, size, shape and weight. These factors enable proteins to be separated by means of electrophoresis, ultracentrifugation, and chromatography. Peptide bonds, as well as certain amino acids, have specific spectral characteristics. Thus we may assay protein concentration based on the amount of light a solution will absorb in the ultraviolet region. There are also a number of analytical methods based on the weight of the protein complex, such as specific gravity and refractive index assays.

Tests Based on Mass or Weight

The procedures based on protein mass or weight assume that organic and inorganic metabolites do not vary from person to person. These procedures are standardized against the Kjeldahl nitrogen reference method with the use of 6.54 as the nitrogen factor. (The Kjeldahl method and the derivation of this 6.54 factor for correcting nitrogen content to protein content is discussed in the next section entitled "Tests Dependent on the Chemical Properties of Proteins.") All of these techniques require careful standardization and exact maintenance of technique.

There is a linear relationship between density or specific gravity and protein concentration of a solution. This may be measured in one of three ways: the falling drop technique, the floatation technique, or the density gradient tube technique. In the falling drop technique, the time it takes for a drop of the protein solution to fall through a standard mixture in a tube of prescribed length (usually 10 cm) is measured. This procedure is sensitive to temperature variations as well as factors which may alter the viscosity of the test solution, such as evaporation. Timing and drop size are also critical.

The best known of the density methods is the floatation technique of Phillips. In this procedure the protein solution is dropped into cupric sulfate solution of known specific gravity. This technique has been used in the past by blood banks to determine if the hemoglobin of the prospective donor was high enough for the individual to donate blood. If a series of cupric sulfate solutions are used which have been standardized against the Kjeldahl nitrogen procedure, then the results may be interpolated and the protein concentration determined. Standard solutions of zinc sulfate or a sodium chloride/picric acid combination may be used in place of cupric sulfate solutions. Changes in viscosity do not affect the floatation techniques as they do falling drop technique, but the factors such as temperature and drop size do apply.

The third technique, that of the density gradient tube, also uses a cupric solution of known specific gravity and is similar to the floatation technique. The temperature, drop size, and timing are not as critical. The protein concentration can be calculated from the following formula:

$$K \times (G - 1.0070) = g/dL$$

The K is similar for most proteins and has been found to be 386. G is the specific gravity of the test solution in which the drop becomes suspended for a few seconds after its initial descent.

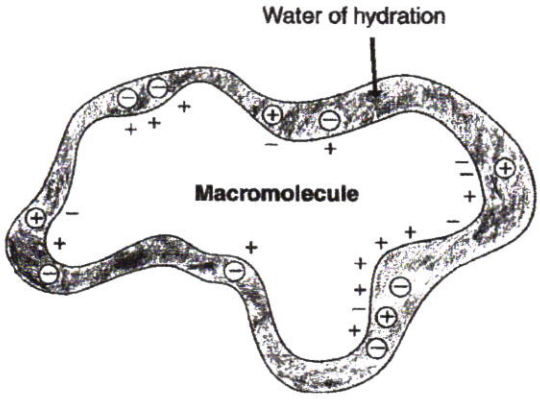
Another way of measuring total protein concentration is to measure the total solid content of the solution. A difference in total solids is a reflection of protein concentration, assuming organic and inorganic metabolites do not vary from person to person. The refractive index (RI) is a ratio of velocities of light in two different media, i.e. air and water. Particles within an aqueous solution affect this ratio. The refractive index may be measured using a total solids (TS) meter (also known as a refractometer). One advantage of this technique is this instrument compensates for changes in temperature. The technique of measuring the refractive index of serum sample is used by many laboratories to validate high or low protein levels determined by automation, preventing the need to re-analyze the sample.

The measurement of refractive index has also been adopted to measure the specific gravity of urine. The object is to measure the total solids as a reflection of the concentration ability of the kidney, and not as a reflection of protein concentration. The presence of protein should be taken into consideration when interpreting the specific gravity of urine measured with a TS meter.

All of these techniques are affected by increased concentrations in lipids, glucose, and nonprotein nitrogen compounds. False elevations can be caused by hemolysis (hemoglobin is a protein), through the use of anticoagulants other than heparin, or in collection by leaving the tourniquet on too long and causing hemoconcentration. Errors can also be caused by improper storage and handling of the standard test solution.

Tests Based on Water Competition Effects

Proteins exist in the form of a colloid in solution. Individual protein molecules are charged because of the presence of ionized chemical groups on their surfaces. These ionized groups attract a layer of water molecules which surround the outer surface of the protein colloid. This layer of water molecules keeps protein in solution (i.e., dissolved in the blood stream). This is illustrated in the diagram below where the macromolecule represents protein.



The attraction of protein molecules for each other is maximal at the isoelectric point (pI). At the pI, there is no net charge on proteins which results in a decreased attraction for water molecules to surround the protein. Since proteins have their greatest attraction for other protein molecules and very little attraction for water molecules at their pI, this is where proteins are least soluble. This is the rationale behind the electrophoretic technique known as isoelectric focusing.

Albumin is water soluble; globulins are not water soluble. With a low concentration salt solution, globulins can be put into solution. This effect is called "**salting in**". "Salting in" results from electrostatic attractions forming between the salt ions and the charged groups on the protein molecule. This serves to decrease the attraction of protein molecules for each other. The cations and anions of the salt bind to the reactive groups on the protein and break the bonds holding the protein-protein complexes together. This allows the individual protein molecules to undergo dissolution (i.e., dissolve in the biological fluid).

The salt concentration of plasma (approximately 0.15M) maintains globulins in solution. As the salt concentration is increased (to a level of approximately 2M), the salt begins to compete with the protein for the water shell which surrounds it. The protein then becomes dehydrated resulting in decreased in solubility. This is called "**salting out**". The level of salt needed to precipitate the various globulins varies with the specific globulin and the type of salt, as well as the pH and temperature. Note, however, that proteins are not necessarily denatured by this technique.

Tests Dependent on pH, Size, and Molecular Weight

Specific differences in physical characteristics allow proteins to be separated by **chromatographic techniques**. Proteins are separated mainly with columns using gel permeation chromatography. Another very common name of gel permeation chromatography is size exclusion chromatography. Gel permeation chromatography separates compounds by their molecular weight. When a mixture of small and large molecules are allowed to pass over small particles of dextran in a glass column, the small molecule and ions diffuse into the gel, and the large molecules pass readily through the column. The dextran used has an accurately controlled pore size; by controlling this size, the separation can be controlled. The technique can be further improved by introducing ion exchange groups. The majority of the functional ion groups are on the surface and are easily exchanged with large molecules which cannot penetrate the closely linked dextran structure. Adsorption is favorable at low salt concentrations. An increase in the salt concentration promotes the dissociation of electrostatic linkages between proteins and the adsorbant. By altering the pH, the number of charges on protein and also on the adsorbant are altered. Thus, elution is carried out by increasing the ionic strength of the salt concentration and changing the pH in a stepwise progression. The four protein fractions that are isolated by this technique include (in order of elution): gamma globulins, beta globulins, albumin, and alpha globulins. Gel permeation chromatography has been used principally for the separation of proteins from smaller molecular weight materials, such as metal ions. It has also been used in the study of isoenzymes.

While chromatography may be used to separate proteins, protein separation is much more commonly achieved by **electrophoresis**. The various types of electrophoresis have previously been considered and this would be a good time to go back and review the basic concepts of each.

As a brief overview of electrophoresis you will recall that electrophoresis can separate protein mixtures on the premise that the individual protein species possess different mobilities when subjected to an electric field. Every molecule possesses an electric charge due to the presence of positively charged groups (i.e., NH_3^+ groups from arginine, lysine, and histidine amino acid residues and terminal amino groups) and negatively charged groups (i.e., COO^- groups from glutamic acid and aspartic acids amino acid residues and terminal carboxyl groups). The net charge dictates the migration characteristics of the species at a given pH.

The acidic and basic properties of proteins in aqueous solution depend on the number of free acidic and basic groups present, on their dissociation constants, and on the spatial distribution of these groups within the molecule (tertiary structure). Since proteins behave as acids or bases depending on the pH of the solution, they are defined as amphoteric. In acidic solutions excess H^+ hampers dissociation of carboxyl groups and most proteins remain at least relatively positively charged. They therefore migrate toward the cathode (negative electrode). In basic solutions, a proton is removed from carboxyl groups and most proteins remain negatively charged and migrate toward the anode.

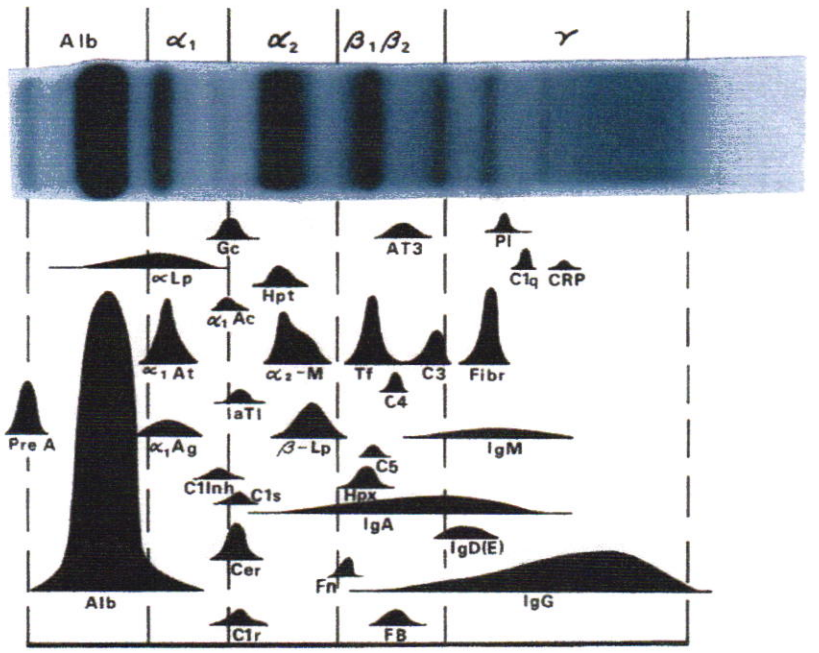
Each protein has a unique pH at which the number of positive and negative charges are equal, i.e., the net charge for the species is zero. At this pH (the isoelectric point, pI) the protein is relatively insoluble in solution and demonstrates no mobility in electrophoretic media unless there is electroendosmotic flow.

The more homogeneous a protein, the more distinct the band it produces on electrophoretic separation - (e.g., albumin, α_1-antitrypsin, and transferrin). Lipoproteins, because of their large and variable size, and immunoglobulins, because of their heterogeneity of charge, produce diffuse bands or hazy areas. Moreover, the further the pI is from the pH of the buffer, the greater the protein mobility and consequently the better the resolution that can be achieved (as long as diffusion is kept to a minimum). The phenomenon of diffusion is a major limiting factor since proteins diffuse continuously within the medium from the time of application to the moment when the proteins are fixed to the electrophoretic medium.

The following is a diagrammatic dissection of a good quality agarose electrophoretic separation demonstrating the complexity of the proteins underlying the rather simple protein bands.

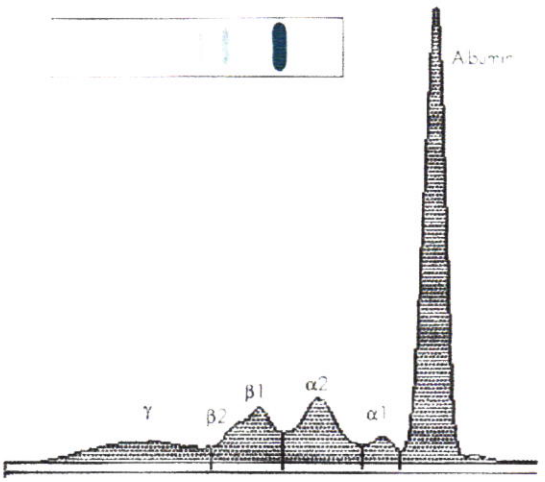
Abbreviations

α_1Ac = α_1-Antichymotrypsin
α_1Ag = α_1-Acid glycoprotein
α_1At = α_1-Antitrypsin
α_2M = α_2-Macroglobulin
α-Lp = α-Lipoprotein
Alb = Albumin
AT3 = Antithrombin III
β-Lp = β-Lipoprotein
Complement Components:
C1q, C1r, C1s, C3, C4, C5 = As indicated
C1Inh = C1 esterase inhibitor
Cer = Ceruloplasmin
CRP = C-reactive protein
Gc = Gc-globulin (vitamin D-binding protein)
FB = Fibrinogen
Hpt = Haptoglobin
Hpx = Hemopexin
Immunoglobulins
IgA, IgD, IgE, IgG, IgM = As designated
IaTI - Inter-α-trypsin inhibitor
PI = Plasminogen
Pre A = Prealbumin
Tf = Transferrin



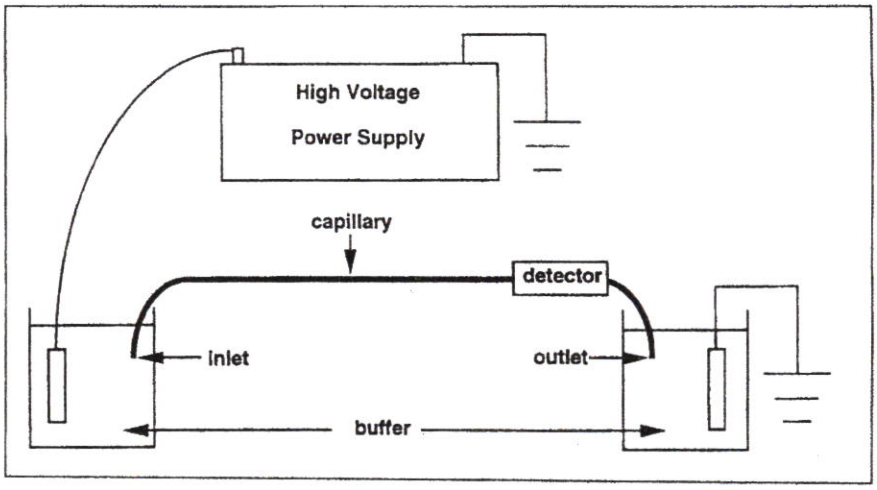
In the diagram above, please disregard the protein abbreviated Fn. Of the proteins noted above, those with known clinical significance will be considered in the following lecture.

A normal serum protein electrophoretic pattern is shown below. Note that when serum is used, two major proteins are missing. These missing proteins include hemoglobin and fibrinogen. Fibrinogen makes up 3 - 6% of the major proteins in the blood stream. However, fibrinogen is consumed in the clotting process causing it not to be detected in serum. If fibrinogen were present, it would appear right the point of application. Recall that the point of application is between the beta globulin fraction and the gamma globulin fraction.



A variation of the routine electrophoretic technique described above is **immunoelectrophoresis (IEP)**. Immunoelectrophoresis is a qualitative technique that is used primarily for the evaluation of immunoglobulins. The theory behind immunoelectrophoresis is that proteins are first separated into five fractions by electrophoresis as described above. Following separation, an antiserum against the protein of interest is allowed to diffuse toward the protein fractions. Precipitin arcs form at the interface between the antiserum and the protein the antiserum is directed toward. These precipitin arcs are visually interpreted. This is illustrated in Tietz page 226, figure 9-5. Tietz indicates that immunoelectrophoresis is being replaced in the clinical laboratory by immunofixation electrophoresis. The reason for this is immunofixation electrophoresis is easier to interpret, as will be illustrated as we begin to look at immunoglobulins later in this unit.

Immunofixation electrophoresis (IFE) has rapidly become the method of choice in the evaluation of immunoglobulins. Below is an illustration of a normal IFE pattern. Please note that, even though the most common sample used for electrophoresis is serum, the sample used for this pattern is plasma. Plasma contains fibrinogen. As noted above, when present, fibrinogen appears at the point of application. This is noted by the small black arrow on the left hand side of the pattern below. Since fibrinogen is not an immunoglobulin, the note beside the small black arrow on the right hand side of the pattern below indicates that there is no reaction in any of the channels where immunoglobulins are evaluated.



Considering the IFE technique, IFE takes advantage of both antibody specificity for antigens and the ability of antibodies to fix (immunoprecipitate) proteins in a gel matrix. IFE is performed using specialized gels in which electrophoresis is performed simultaneously in six adjacent lanes for each specimen. Following separation, a template cutout is placed on the gel such that it isolates each of the six electrophoretic lanes. The left lane is then overlaid with a general protein fixative. Each of the second through the sixth lanes is overlaid with respective antisera against IgG, IgA, IgM, kappa light chains, and lambda light chains. In lanes 2 - 6, the protein of interest is precipitated and fixed in the gel matrix. Any proteins not fixed are washed away by the addition of a buffer rinse. Following washing, the gel is stained for visualization.

IFE is most commonly used on serum, but it can also be used to identify immunoglobulins in urine and CSF. It is important to note that both urine and CSF samples must be concentrated prior to IFE.

Capillary electrophoresis (CE) is the newest type of electrophoresis used in the analysis of proteins. This technique is changing the way many proteins and polypeptides are studied in diagnostic and forensic laboratories because it is rapid, sensitive, and adaptable to automation. Clinical laboratories performing electrophoresis in high volume are replacing cellulose acetate and agarose-gel manual systems with automated CE systems. CE also promises to be useful for the evaluation of hemoglobin variants and the quantitation of hemoglobin A_2, hemoglobin F, and glycated hemoglobins.

Capillary electrophoresis is performed in a long, thin capillary that may or may not contain a support medium such as a gel. A diagram of a typical experimental setup is shown below.

Typical dimensions for the capillaries are 50 to 100 cm in length and 50 to 150 mm in diameter. Because the volume inside the capillary is quite small, and the sample must be a very small fraction of the inside volume, typically only nanoliter (nL) quantities are injected. Theory indicates that resolution of proteins is dependent upon only the applied electric field strength and that column length does not influence separation efficiency. Column length does, however, affect analysis time. Therefore, the ideal situation would be to apply as high a voltage as available to a capillary system that is as short in length as possible. The very high surface/volume ratio of the capillary allows for very effective heat dissipation, so very high voltage may be used without denaturing the protein being studied. This high field strength results in rapid separation, with high resolution being achieved in a short time. In addition, capillary zone electrophoresis is very amenable to automation.

In unmodified silica glass capillary tubes, surface charges are abundant so electroendosmosis may be a significant consideration for capillary electrophoresis. For this reason, modification of the column wall to minimize residual charges is performed, which greatly reduces this effect.

A disadvantage of capillary electrophoresis is that the very small sample volume (nL) places stringent demands on detection techniques. However, as the individual proteins are separated into very narrow bands, they pass by a detector window in the capillary and are immediately read by a detector before they have a chance to become more diffuse and difficult to detect. The most common type of detector used is a UV detector. The amount of light absorbed at 215 nm by peptide bonds is directly related to protein concentration. Serum protein electrophoresis patterns produced using capillary electrophoresis are very similar to those produced using cellulose acetate or agarose gel. The high resolution of CE, however, shows distinct transferrin and complement zones within the beta region, something typically not seen with other types of electrophoresis.

Isoelectric focusing (IEF) is another variation of electrophoresis used in the study of proteins. IEF is a high resolution technique that separates proteins on the basis of their isoelectric point (pI). The membrane used for this electrophoretic separation contains a pH gradient, i.e., the pH of the membrane gradually changes. This gradient is created by the use of amphoteric polyaminocarboxylic acids which, when properly prepared, will create buffered zones in the electrophoretic medium with stable but slightly differing pHs. Gradients currently available typically range from pH 3 to 10.

To carry out IEF, the sample containing the proteins of interest is placed on the IEF membrane and an electrical current is applied. The proteins in the sample will migrate in the electrical field until they reach the area of the membrane where the pH of the membrane equals the pI of the particular protein. At this point, migration stops (because the protein is now electrically neutral) and the protein is focused in a very narrow band. As long as the pH gradient and electric field are present, no further migration (or diffusion) will occur. Proteins with pI differences of as small as 0.02 pH units have been separated by IEF.

Tests Dependent on the Chemical Properties of Proteins

The normal reference range for Total Serum Protein is 6.0 - 8.0 g/dL. For ambulatory individuals, the normal reference range is typically increased 0.5 g/dL to 6.5 - 8.5 g/dL.

The normal reference range for albumin is 3.5 - 5.2 g/dL.

Once total serum protein and albumin concentrations have been determined, the globulin concentration can be calculated by the following equation:

Total protein – albumin = globulins

This is the usual way of determining the concentration of globulins. There is a procedure, however, for measuring total globulin concentration if necessary.

A/G ratio - may be calculated , but no known clinical significance so rarely done.

Introduction

The majority of chemical tests for proteins are nonspecific. They do not test for the protein itself, but rather for specific elements of groups within the protein complex. All proteins are composed of nitrogen, carbon, and oxygen atoms. The nitrogen atom sets the proteins apart from the two other body building blocks: lipids and carbohydrates.

All total protein methods are based on two assumptions:

1. All proteins contain 16% nitrogen
2. Each protein reacts chemically like every other protein

Theses assumptions may not always be true, but methods based on these assumptions are considered accurate.

1. Kjeldahl - very tedious, rarely used, but still considered reference method.

The classic method for determining protein is the Kjeldahl method, which was also the first total protein method developed. This method is based on the determination of the nitrogen content of the protein. This method is still used today as a reference method in the standardization of all other protein procedures.

Nitrogen $\xrightarrow{\text{acid}}$ NH_4^+ (ammonium ion)

NH_4^+ $\longrightarrow$ NH_3 (Ammonia) measured by titration **with HCL**

Compensation for nonprotein nitrogen constituents must be made. This can be done in one of two ways: trichloroacetic acid or tungstic acid can be used to precipitate the protein. The precipitate can then be used to determine the protein nitrogen content, or a simultaneous assay for the nonprotein nitrogen (on filtrate) and total nitrogen (on the serum sample) can be performed. The difference between the two represents protein nitrogen.

Although the Kjeldahl procedure has been accepted by most as the reference standardization method, there is an inherent problem in converting the protein nitrogen content to protein content. It was previously assumed the 16 % of the protein mass is nitrogen and the factor 6.25 was used to convert nitrogen content to protein concentration.

$$\frac{protein}{nitrogen} = \frac{100\%}{16\%} = \frac{1.00}{0.16}$$

$$\frac{protein}{nitrogen} = 6.25$$

$$protein = 6.25 \times nitrogen$$

No one knows why 16 % was chosen. Total protein is a heterogenous mixture of component proteins. The nitrogen content of the various fractions is different. There is an even greater disparity in relating nitrogen content to protein values in pathological conditions. It has also been found that the results with the Kjeldahl procedure will vary with the length of each step in the procedure.

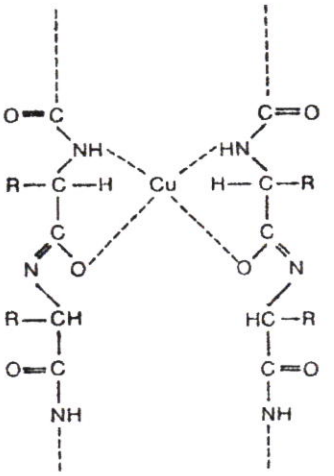
The nitrogen content of the different protein fractions varies. Upon electrophoretic fractionation and subsequent determination of nitrogen content, the factors for albumin, alpha, beta, and gamma globulins have been found to be 6.53, 6.63, 6.78, and 6.52 respectively. An average figure of 6.54 (i.e., an average nitrogen content of 15.3%) for correcting nitrogen content to protein content has been suggested.

2. **Biuret - Based on presence of peptide bonds; simple assay; easy to automate**

Nitrogen + Cu^{++} —Alkaline→ Violet complexes

Cupric ions react with peptide bonds in proteins to form a colored complex which can be spectrophotomically measured at 540nm

- Biuret name came from organic compound biuret which also reacts with Cu^{++}. Any reactions with Cu^{++} and peptide linkages named a biuret reaction
- at least two peptide bonds required for a reaction to take place, i.e.tripeptide
- Not sensitive enough for extremely low protein levels; does not work well for CSF or urine
- Most commonly used total protein method



Biuret Linkage

The intensity of color is proportional to the number of peptides undergoing the reaction. The more protein present, the more peptide bonds available for reaction. Note however that there is no correlation between the color produced and the molecular weight of the protein or polypeptide.

- Highly icteric and lipemic sera will cause false elevations of the total protein

3. **Refractive Index:** based on the assumption that increased total protein concentration with increase refractive index of serum.

- assumes electrolytes and non-protein compounds in serum don not vary appreciably from serum to serum so difference in refractive index is due to protein concentration.
- large false positive errors will occur if glucose or urea are abnormally high
- icteric and lipemic specimens will falsely increase
- most useful if only looking for a fast estimate of protein concentration

4. **Dye-binding**: based on proteins ability to bind various dyes

- affinity with which they bind varies
- most often used with serum protein electrophoresis to stain separate protein fractions (stains used such as Ponceau S, Coomassie blue, amido black 10B)
- Albumin has a much greater binding power than globulins so procedure used primarily for albumin measurement.
  - Bromcresol-green (BCG) dye-binding - Most common albumin method and very easy to automate.

    Albumin + BCG Alb/BCG complex
    Read at 640nm
  - Methyl orange and [2-(4'-hydroxyazobenzene)-benzoic acid] or HABA, other methods currently used
  - The bromcresol green procedure has the least interference from pigments and is typically the dye-binding method of choice for measuring albumin.
  - Albumin falsely increased with heparin
  - falsely decreased with salycilates

5. **Lowry-copper phenol method**. This method has been developed for body fluids with low concentrations of protein by combining the biuret and Folin-Ciocalteau reactions. By including cupric ions in the phenol reaction, the sensitivity is increased three to 15-fold. The resultant sensitivity is about 100-fold that seen with the biuret reaction. Two reactions are involved. One is that of the protein and cupric ion in an alkaline medium and the other is the reduction of the phosphotungstic and phosphomolybdic acids to molybdenum blue and tungsten blue by the copper protein complex and the phenolic side chains of the protein. About 75 percent of the color depends on the cupric ion.

6. **Immunochemical methods:** used to detect individual plasma proteins.

a. Nephlometry - specific antibodies used to differentiate proteins, as they bin and form ag/ab complexes which will increase light scatter., i.e. Beckman Array
These two techniques have previously been considered and this would be a good time to go back and review the basic concepts of each

b. RID - Radial immunodiffusion - Ab is in gel - sample applied to gell will migrate out in gel and form a precipitive . Diameter of precipitant ring directly proportional to protein.

7. **Separation of plasma proteins:** Used to determine the true cause of a protein disorder. Identifies and quantitates specific proteins.

a. Serum protein electrophoresis - discussed earlier in this lecture
b. Immunofixation - discussed earlier in this lecture
c. Salt fractionation - used to separate albumin from globulins
   - globulins will precipitate out in high salt concentrations leaving albumin in supernatant; can then be measured using TP procedure i.e. biuret
   - labor intensive - more direct methods for albumin available.
d. Chromatography - separates out proteins between two phases .
   - affinity chromatography - protein in liquid pass over solid support(solid & liquid phase) and adsorb to solid phase at different places
e. Ultracentrifugation - similar to lipid method
   - separates proteins based on individual densities